BIOCHEMICAL EVIDENCE FOR INDUCTION BY POLYOMA VIRUS OF REPLICATION OF THE CHROMOSOMES OF MOUSE KIDNEY CELLS

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Abstract.—It was shown earlier that in cultures of contact-inhibited mouse kidney cells infection with polyoma virus induces replication of the chromosomal DNA. In this paper we present evidence, based on analyses of isolated chromatin, that, parallel with and as a consequence of the virus-induced synthesis of cellular DNA, the chromosomal proteins are synthesized in the proportions characteristic of normal chromosome replication. These results are compatible with the hypothesis that polyoma virus activates (or derepresses) a regulatory system of the host cell which controls initiation of chromosome replication.

Introduction.—When primary mouse kidney (MK) cell cultures become confluent, most cells stop synthesis of cellular DNA and mitosis. Infection with polyoma (Py) virus induces renewed synthesis of cellular DNA,[1, 2] in which essentially the whole of the chromosomal DNA participates but which is not followed by mitosis.[3a–c] Since replication of chromosomal DNA during the mitotic cycle of eukaryotic cells is accompanied by synthesis of histones, [4a–c] we investigated whether Py-induced replication of cellular DNA is similarly accompanied by synthesis of histones. The results reported in this paper show that this is the case and suggest, further, that infection induces also the synthesis of other chromosome-associated proteins.

Materials and Methods.—MK cell cultures were prepared according to Winocour[5] in plastic Petri dishes (88 mm diameter). One to two days after reaching confluence they were infected at 37°C with 0.4 ml of the same strain of wild-type Py virus (*ca.* 10^9 PFU/ml) as used previously in this laboratory. After adsorption of the virus for 2 hr, the cultures were washed once with Eagle's medium and covered with 10 ml of serum-free medium. Control cultures were mock-infected and thereafter treated like Py-infected cultures. The concentration of L-lysine in the Eagle's medium used was 0.8 μM/ml. In experiments with 5-fluorodeoxyuridine (FUdR), the cultures were infected and washed in the same way and then covered with 10 ml of serum-free medium containing FUdR at a concentration of 6×10^{-5} *M*. As judged by autoradiography, DNA synthesis is inhibited under these conditions in more than 99% of the Py-infected cells.[6] The inhibition of DNA synthesis was reversed at the times indicated in *Results* by addition of deoxythymidine (TdR; 5 μg/ml) to the FUdR-containing medium. Details of the materials and procedures used are described elsewhere.[6]

Isolation and fractionation of chromatin: Cultures were washed with ice-cold Eagle's medium and the cells were detached, using a rubber scraper, into 5 ml of the same medium. The suspension of cells from 2 or 3 Petri dishes (see *Results*) was pooled, the cells were sedimented (2500 *g* for 15 min at 4°C), resuspended in 10 ml of an ice-cold 0.15 *M* NaCl solution (pH 7–8), and again sedimented. The cell pellet was kept frozen at −80°C until used. Chromatin was isolated and fractionated with a procedure based on that of Dingman and Sporn,[7] which was developed for HeLa cells and is described in detail elsewhere.[8] The cell pellet was thawed by addition of 2 ml of a solution containing NaCl, Na-EDTA, and Triton X-100 at concentrations of 80 mM, 20 mM, and 1%, respectively

(pH 7.2). An aliquot of this suspension was used for determination of total cellular DNA.[8] The resuspended cell pellet was then subjected to 6 cycles of homogenization (Teflon-glass homogenizer, Bellco, USA; 1000 rpm; 1 min) in the same solution followed by centrifugation in the homogenizer tube (2500 *g* for 15 min). The final pellet containing the chromatin was suspended in 1 ml of 0.2 mM Na-EDTA (pH 7.2) by gentle homogenization. All procedures were carried out at 2–4°C. From aliquots of the final chromatin suspension, DNA, RNA, histones, and nonhistone proteins were extracted as described in detail elsewhere.[8] DNA was determined by the diphenylamine method[9] using calf-thymus DNA (Calbiochem) as standard, RNA by spectrophotometry at 260 mμ, and protein by the method of Lowry *et al.*,[10] with crystalline pancreatic RNase (Calbiochem) as standard.

Radioactivity in DNA, histones, and nonhistone proteins was measured in 10 ml of Bray's scintillant solution.[11] Appropriately quenched ^{3}H and ^{14}C standards were used to adjust the counting channels of a liquid scintillation counter (Nuclear-Chicago Unilux-II) so that ^{14}C and ^{3}H could be discriminated in the same sample. The data are not corrected for counting efficiency, but counting background is deducted.

Electrophoretic analysis of histones: Extracts of chromatin (in 0.2 *N* HCl) were concentrated to a volume of 50–100 μl in collodion sacs (Sartorius, Germany) surrounded by Sephadex G-200, then dialyzed against electrophoresis buffer containing 1 *M* sucrose (Merck). During these procedures 98% of the histones were recovered, as determined by radioactivity.

Disc electrophoresis in polyacrylamide gel was performed at 4°C essentially according to Reisfeld *et al.*[12] The acrylamide monomers were recrystallized before use;[13] acrylamide concentrations were 15% (main gel) and 3.3% (stacking gel); both gels contained 6 *M* urea.

After electrophoresis, the stained gels were analyzed in a Joyce-Loebl microdensitometer. They were then frozen in a Dry Ice-acetone mixture and sliced into discs 1.5 mm thick. The discs were dissolved by using the method of Moss and Ingram;[14] radioactivity was determined after addition of 10 ml. of scintillant solution. Under the conditions used, 75–90% of the radioactivity loaded on the gels was recovered in the five major histone bands (see Fig. 1).

L-lysine-U-^{14}C (spec. act. 300 mc/mM) and DL-lysine-4,5-^{3}H (spec. act. 8.2 c/mM) were obtained from the Radiochemical Centre, England, and TdR-methyl-^{3}H (spec. act. 6.7 c/mM) from New England Nuclear Corp., Boston. FUdR was a gift from Hoffmann-La Roche, Basel.

Results.—Under the conditions used, confluent MK cultures contain 5–7 × 10^{6} cells, and, as determined chemically, 30–50 μg of chromosomal DNA. Feulgen-microspectrophotometry shows that in the majority of the cells the mitotic cycle is arrested prior to the duplication of the chromosomal DNA.[3a, b] However, these confluent cultures also contain a small proportion of fibroblast-like cells which are not contact-inhibited and thus continue to undergo DNA synthesis and mitosis.[3a]

The time course of infection with Py virus is very asynchronous: a small number of cells (<1%) start cellular and viral DNA synthesis around 12 hours after infection. Thereafter the number of DNA-synthesizing cells increases rapidly and reaches a maximum, corresponding to 60–80% of the cells, around 30 hours after infection.[3a, 3b, 6]

(1) *Chromatin composition of uninfected confluent mouse kidney cells:* Chromatin of MK cells contains the chromosomal DNA, with which are associated the histones, nonhistone proteins, and a very small quantity of RNA. By the method used, about 90% of the total cellular DNA is recovered in the chromatin

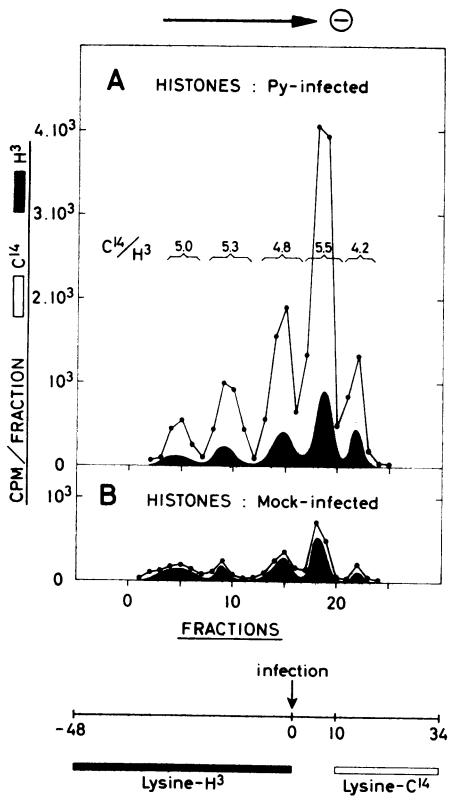

FIG. 1.—In Py-infected confluent MK cultures the individual histone fractions are synthesized in similar relative proportions as in uninfected, actively growing MK cells. The figure represents a comparative analysis by polyacrylamide gel electrophoresis of histones from *Py-infected* (*A*) and *mock-infected cultures* (*B*). To actively growing cultures lysine-^{3}H (7 μc/ml) was added 1 day after plating and was left until 1 day after the cultures had become confluent. Three cultures were then infected with Py virus and three cultures were mock-infected. After 10 hr, the medium was replaced on all cultures by fresh medium containing lysine-^{14}C (0.3 μc/ml). Chromatin was isolated 34 hr, after infection; the histones were extracted and separated on polyacrylamide gels, which were stained and processed for analysis of radioactivity as described in *Materials and Methods*. The ratio ^{14}C/^{3}H in the samples prior to electrophoresis was 4.7 for sample *A* and 1.0 for sample *B*. The values for ^{3}H are corrected for approximately 25% of the total ^{14}C radioactivity which appeared in the ^{3}H-counting channel.

(Table 1). As determined chemically, the mass ratios to DNA of histones, of nonhistone proteins, and of RNA are very close to those reported for chromatin isolated from a number of other eukaryotic cell types.[8, 15]

Polyacrylamide gel electrophoresis of the histones from uninfected MK cells shows a pattern with five major bands (Fig. 1) which is not detectably different from those of histones from HeLa cells[8] and from calf-thymus cells.[15] From the very low level of RNA and the absence of ribosomal proteins (which, if present, would have been detected by polyacrylamide gel electrophoresis of acid-extractable proteins), we conclude that the chromatin used in our experiments is essentially free from contaminating ribosomes or ribosomal subunits.

(2) *Evidence for induction by polyoma virus of synthesis of histones and other chromosome-associated proteins:* (a) *Incorporation studies using lysine-^{14}C* show that until about 15 hours after infection, incorporation of lysine-^{14}C into histones is essentially the same in Py-infected and in mock-infected cultures. Thereafter, the rate of incorporation markedly increases in Py-infected cultures (Fig. 2). This observation is in agreement with the findings of Shimono and Kaplan,[16] but is at variance with those of Gershon *et al.*[17] The rate of incorporation of lysine-^{14}C into histones increases in Py-infected cultures at essentially the same time as the rate of incorporation of TdR-^{3}H into DNA (Fig. 2). The apparent paradox, that the rate of incorporation of radioactive TdR decreases around 30 hours after in-

TABLE 1. *Composition of chromatin from uninfected confluent mouse kidney cultures.**

Recovery of cellular DNA in chromatin†	Mass Ratio in Chromatin‡		
	Histones/ DNA	Nonhistone proteins/ DNA	RNA/ DNA
93 ± 4%	1.21 ± 0.07	0.58 ± 0.04	0.03 ± 0.003

* Mean and standard deviation of 5 independent preparations, each from 10–15 pooled cultures.
† To label cellular DNA, uninfected MK cells were grown from the time of plating to confluence (3 days) in medium containing TdR-^{3}H (2 μc/ml). The radioactivity recovered in the DNA of chromatin was compared with that in the total cellular DNA. Quantitative chemical determinations gave comparable results (Hancock, unpublished).
‡ From chemical determinations (see *Materials and Methods*).

fection when the number of cells synthesizing DNA is at a maximum (as judged by autoradiography), is due to such factors as a decreased rate of cellular DNA synthesis, an increase in the endogenous pool of thymidine-5′-phosphates, and the detachment of Py-infected cells from the Petri dishes during the later stages of infection.[3a] Similar factors may be responsible for the observed decrease at around 30 hours p.i. in the rate at which lysine-^{14}C is incorporated into histones. Since we have not demonstrated that the relative extent of utilization of exogenous ^{14}C-lysine is the same in Py-infected and uninfected cultures, lysine incorporation cannot with certainty be related quantitatively to *de novo* synthesis of histones.

(b) *Chemical determinations* show that at around 30 hours after infection, the chromatin of Py-infected cultures contains 50–60 per cent more DNA than the

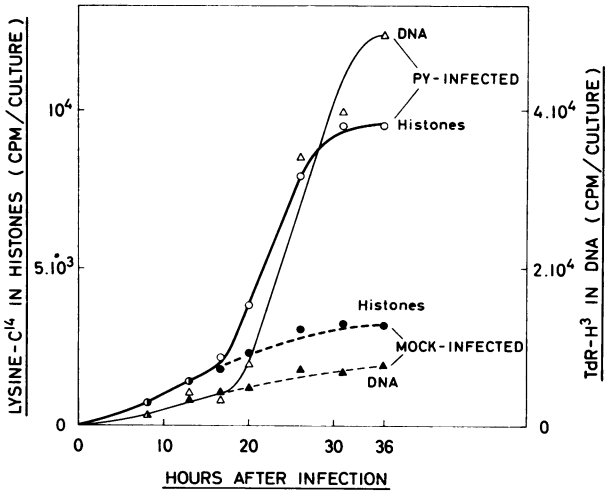

FIG. 2.—Infection with Py virus leads to stimulation of incorporation of lysine-^{14}C and of thymidine-^{3}H into histones and DNA, respectively, of the chromatin of confluent MK cultures. Py-infected and mock-infected cultures were covered (2 hr after infection) with 10 ml Eagle's medium containing TdR-^{3}H (2.5 μc/ml), nonradioactive TdR (2 μg/ml), and lysine-^{14}C (0.06 μc/ml). Each point represents the average of the amount of radioactivity present in the chromatin of two pooled cultures. Sedimentation velocity analyses show that around 30 hr after infection approximately 10% of the radioactivity in DNA extracted from chromatin is present as circular Py DNA.[3c] The relative amount of Py DNA is, however, lower since its specific radioactivity is considerably higher than that of cellular DNA.[2]

TABLE 2. *Evidence that in confluent mouse kidney cultures Polyoma virus induces synthesis of both cellular DNA and of chromosomal proteins.*

Chromatin derived from:	Hr after infection	Quantity (μg) in Chromatin/Culture DNA	Histones	Nonhistone/ proteins	Mass Ratio Histones/ DNA	Nonhistone proteins DNA
Mock-infected controls*	(2)	28 ± 2	36 ± 7	15 ± 4	1.28	0.54
Mock-infected controls†	(32)	30 ± 3	39 ± 6	17 ± 3	1.30	0.57
Py-infected*, †	32	45 ± 3	59 ± 5	29 ± 4	1.31	0.64
Py-infected + FUdR‡	32	30	41	18	1.36	0.60

* Eight cultures were divided into 4 pairs, from which chromatin was isolated separately. The values represent the mean and standard deviations of chemical determinations on these 4 replicate preparations.

† The Py-infected cultures have lost approximately 20% of the cells originally present.

‡ FUdR (6×10^{-5} *M*) was added 2 hr after infection. Four cultures were divided into 2 pairs; the values are the means of these 2 duplicate preparations.

chromatin of mock-infected cultures (Table 2), a difference comparable to that found by earlier determinations of total cellular DNA.[3a] (It should be pointed out that at all times after infection, circular Py DNA accounts for less than 10% of the total DNA in Py-infected cultures.[3a] The amounts of histones and nonhistone proteins in the chromatin of Py-infected cultures show comparable increases, resulting in maintenance of apparently constant mass ratios with respect to DNA.

(c) *Polyacrylamide gel electrophoresis of the histones of polyoma-infected mouse kidney cells* shows a pattern which is indistinguishable from that of histones of uninfected cells. The relative incorporation of lysine-^{14}C into the individual histone bands of Py-infected cultures is essentially the same as that observed in uninfected growing cultures and also in mock-infected confluent cultures (Fig. 1). As judged by this method, the individual histones are synthesized in Py-infected cells in essentially the same proportions as in uninfected, normally replicating cells.

(3) *Inhibition by FUdR of polyoma-induced histone synthesis:* The addition of FUdR (6×10^{-5} *M*) to the culture medium immediately after the adsorption of Py virus (2 hr after infection) inhibits the synthesis of cellular and viral DNA in more than 99 per cent of the cells. In cells where Py-induced DNA synthesis is inhibited, little if any capsid protein is synthesized.[6] Quantitative chemical determinations (Table 2) show that the amounts of histone and DNA in the chromatin of Py-infected cultures which were maintained in the presence of FUdR do not increase above the levels found in chromatin of mock-infected cultures. Similarly, in the presence of FUdR, the rate of incorporation of lysine-^{14}C into histones remains at the low level of mock-infected controls (Fig. 3). The significance of this residual incorporation of lysine-^{14}C into histones in the apparent absence of cellular DNA replication[4c, 18] remains unknown.

By the use of FUdR the onset of Py-induced DNA synthesis can be synchronized:[3a, 3c, 6] In the presence of FUdR the early events of the infective cycle take place just as they do in parallel cultures infected in the absence of the inhibitor.

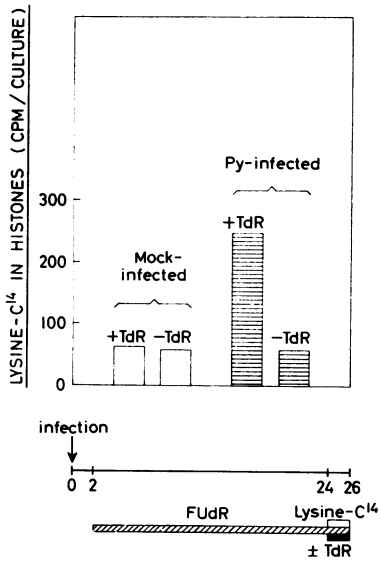

FIG. 3.—Evidence that DNA synthesis is prerequisite for Py-induced stimulation of incorporation of lysine-^{14}C into histones. Four Py-infected and four mock-infected cultures were covered with medium containing FUdR (6×10^{-6} *M*) at 2 hr after infection. From 24 to 26 hr after infection lysine-^{14}C (0.3 μc/ml) was added to the FUdR-containing medium. At the same time TdR (5 μg/ml) was added to the medium of two Py-infected and two mock-infected cultures; under these conditions the majority of the Py-infected cells start DNA synthesis synchronously upon addition of TdR.[3a, 3c, 6]

Therefore, if TdR (which specifically reverses the inhibitory effect of FUdR) is added around 30 hours after infection, when most infected cells are activated, Py-induced DNA synthesis (cellular and viral) starts synchronously. As judged by microspectrophotometry, in the majority of cells the chromosomal DNA has undergone one cycle of replication by seven hours after the addition of TdR.[3b] Figure 3 shows that synchronized DNA synthesis is accompanied by a markedly increased rate of incorporation of lysine-^{14}C into histones. If, some hours after the addition of TdR, DNA synthesis is inhibited again by removal of TdR and addition of FUdR,[3b] incorporation of lysine-^{14}C into histones fairly rapidly decreases (R. Hancock, unpublished results). These results suggest that Py-induced DNA synthesis is prerequisite for the observed stimulation of histone synthesis in Py-infected cultures.

(4) *Increased synthesis of chromosome-associated nonhistone proteins in polyoma-infected mouse kidney cultures:* In addition to the increase in the quantity of nonhistone proteins in the chromatin of Py-infected cultures, as determined chemically (Table 2), the rate at which lysine-^{14}C is incorporated into these proteins is increased to an extent which is comparable to that observed for the incorporation of lysine-^{14}C into histones. Since the nonhistone proteins of chromatin have not yet been well characterized, and because of the uncertainty whether they are all true constituents of the chromatin,[7] they were studied here in less detail than the histones. Furthermore, the possibility that this fraction may include some viral capsid protein cannot be ruled out.

Discussion.—The results reported in this paper suggest that Py-induced replication of the chromosomal DNA in mouse kidney cells is accompanied by synthesis of the histones and of the chromosomal nonhistone proteins in essentially the same proportions as during the normal mitotic cycle.

During chromosome replication in growing eukaryotic cells (phase *S*), histone

synthesis is dependent on cellular DNA replication, which appears to be prerequisite for the transcription of messenger RNA coding for histones.[19] An analogous situation apparently holds for Py-infected MK cells: inhibition of Py-induced DNA replication by FUdR results also in inhibition of the synthesis of the chromosomal proteins. It appears, therefore, that Py-induced synthesis of chromosomal proteins is dependent on virus-induced replication of cellular DNA.

Experimental evidence reported earlier showed that infection of contact-inhibited MK cells with Py virus leads to the activation of the cellular DNA-synthesizing apparatus.[3a, 3b, 6] The present results considered together with those published earlier are compatible with the hypothesis that Py virus activates (or depresses) a regulatory system of the host cell which controls initiation of chromosome replication.

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Abbreviations: FUdR, 5-fluorodeoxyuridine; MK, mouse kidney; PFU, plaque-forming units; Py, polyoma; TdR, deoxythymidine.

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